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Stat3 is essential for neuronal differentiation through direct transcriptional regulation of the Sox6 gene

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ABSTRACT

The transcription factor Signal Transducer and Activator of Transcription 3 (Stat3) functions in various cellular processes including neuronal differentiation. We show that the SRY-box containing gene 6 (Sox6) gene, important for neuronal differentiation, is a direct target gene of Stat3. We demonstrate that in response to ligand stimulation, Stat3 binds to the Sox6 promoter and induces its expression. Furthermore, Stat3 is activated and Sox6 is induced during neuronal differentiation of P19 cells in the absence of exogenous ligand treatment. Moreover, using an RNA interference approach, we show that Stat3 is required for Sox6 expression during neuronal differentiation.

1. Introduction

The transcription factor Signal Transducer and Activator of Transcription 3 (Stat3), a member of the STAT family of transcription factors, is activated through tyrosine phosphorylation in response to cytokines that bind to the gp130 cell surface receptors including leukemia inhibitory factor (LIF), interleukin-6 (IL-6), and oncostatin M (OSM) [1]. Following receptor activation, phosphorylated Stat3 dimers enter the nucleus to regulate transcription of Stat3 target genes [1]. Stat3 mediates various cellular processes including proliferation, postnatal survival, oncogenesis, and maintenance of pluripotency of murine embryonic stem (ES) cells [2–4]. Stat3 also promotes neuronal differentiation of ES cells and P19 cells, and inhibition of the Stat3 signaling pathway blocks neuronal differentiation in these cells [5–7]. However, the exact mechanism by which Stat3 promotes neuronal differentiation is not well defined.

One approach to understand how Stat3 promotes neuronal differentiation is through analysis of Stat3 target genes. We recently identified SRY-box containing gene 6 (Sox6) as a potential Stat3 target gene in a genome-wide ChIP screen [8]. Similar to Stat3, it has been shown previously that Sox6 is required for neuronal differentiation [9,10]. Transcriptional regulation of Sox6 by Stat3 could be a possible mechanism by which Stat3 promotes neuronal differentiation.

In this report, we demonstrate that Stat3 binds to the promoter of the Sox6 gene in response to cytokine treatment. Furthermore, Stat3 increases Sox6 expression in response to cytokine stimulation and during neuronal differentiation independent of exogenous ligand in P19 cells. Finally, utilizing an RNAi technique we show that Sox6 expression is dependent on Stat3 during neuronal differentiation.

2. Materials and methods

2.1. Cell culture and antibodies

NIH3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen). P19 cells were provided by Dr. Richard Cerione (Cornell University College of Veterinary Medicine, Ithaca, NY) and maintained as described previously [9,10]. Induction of P19 neuronal differentiation was performed as described previously [9,10] with 500 nM retinoic acid (Sigma). Antibodies used in this study were described previously [8,11]. Cells were treated with 25 ng/ml recombinant mouse OSM (R&D Systems) or 10 ng/ml LIF (Chemicon International).

Abbreviations: Stat3, signal transducer and activator of transcription 3; RNAi, RNA interference; Sox6, SRY-box containing gene 6; LIF, leukemia inhibitory factor; OSM, oncostatin M

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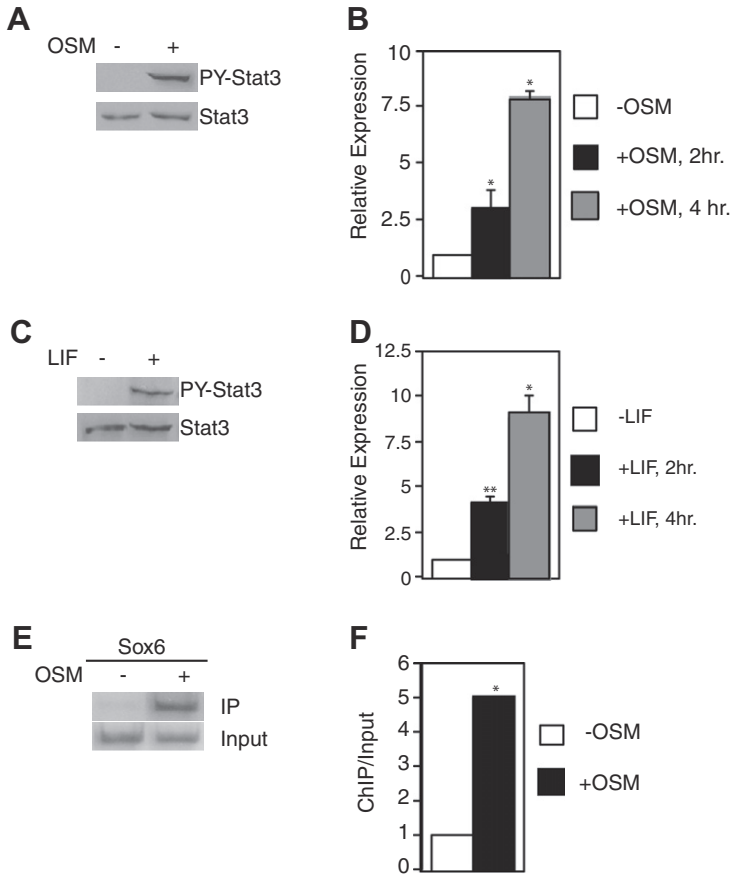

Fig. 1. Sox6 is a direct target gene of Stat3. (A and C) NIH3T3 cells were treated with OSM or LIF for 15 min. Whole cell extracts were used for Western blot analyses with antibodies against Stat3 phosphorylated on tyrosine 705 (PY-Stat3) or total Stat3. (B and D) NIH3T3 cells were treated with OSM or LIF for 2 or 4 h. Quantitative real-time RT-PCR and statistical analyses were performed for Sox6 expression as described in Section 2. (E) NIH3T3 cells were either treated with OSM for 30 min or left untreated and ChIP assays were performed with a Stat3 antibody and sequence-specific PCR primers for the Sox6 promoter. (F) Results from (E) were quantitated with a phosphorimager and expressed as ChIP/input with the untreated sample set at 1.

2.2. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously [8]. Primers for the Sox6 promoter were: 5′ AGTCAGAAGGCGGTGTTAGG and 5′GTAGTTGTGGGCGGAGAAGA.

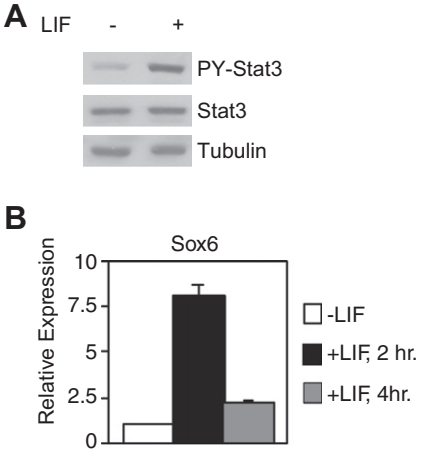

Fig. 2. Sox6 expression is induced by LIF in P19 cells. (A) P19 cells were cultured in growth medium and treated with LIF for 15 min. Whole cell extracts were analyzed by Western blot analyses with antibodies against Stat3 phosphorylated on tyrosine-705 (PY-Stat3), total Stat3 or tubulin. (B) P19 cells were treated with LIF for 2 or 4 h. Quantitative real-time RT-PCR and statistical analyses were performed for Sox6 as described in Section 2.

2.3. Quantitative real-time RT-PCR and statistical analysis

Quantitative real-time RT-PCR was performed as described previously [11]. Results represent the averages of at least three independent experiments standardized to GAPDH with untreated samples set at 1 (Figs. 1 and 2) or represented as fold induction in cells cultured in retinoic acid compared to cells cultured without retinoic acid (Figs. 3–5). For indicated experiments, a Student's *t*-test was performed where (*) represent *P* values less than 0.01, (**) represent *P* values less than 0.05, and no asterisk represents no statistically significant difference compared to indicated control values. *P* values were obtained for treated cells compared to untreated cells (Figs. 1 and 2); cells cultured in retinoic acid compared to cells cultured without retinoic acid (Fig. 3); and Stat3 knockdown cells compared to control cells (Fig. 4). For Fig. 5, data obtained for either control cells or Stat3 knockdown cells were compared to day 2 values. Primers for Sox6 were: 5′ GGCAACTCTCCACCATGATT and 5′ CTTGCGATCTGTTCTTGCTG; Nestin: 5′ CTGCAGGCCACTGAAAAGTT and 5′ AGGTGTCTGCAAGCGAGAGT; N-cadherin: 5′ GAAGGATGTGCACGAAGGAC and 5′ GCTCTGCAGTGAGAGGGAAG; neuronal cell adhesion molecule (NCAM); 5′ CTGTGTCAAGTGGCAGGAGA and 5′ GTCGATGTTGGCGTGTAGA and GAPDH: 5′ AGACACCAGTAGACTCCACG and 5′ ACGACCCCTTCATTGACC.

2.4. RNAi

RNAi was performed as described previously [8,11].

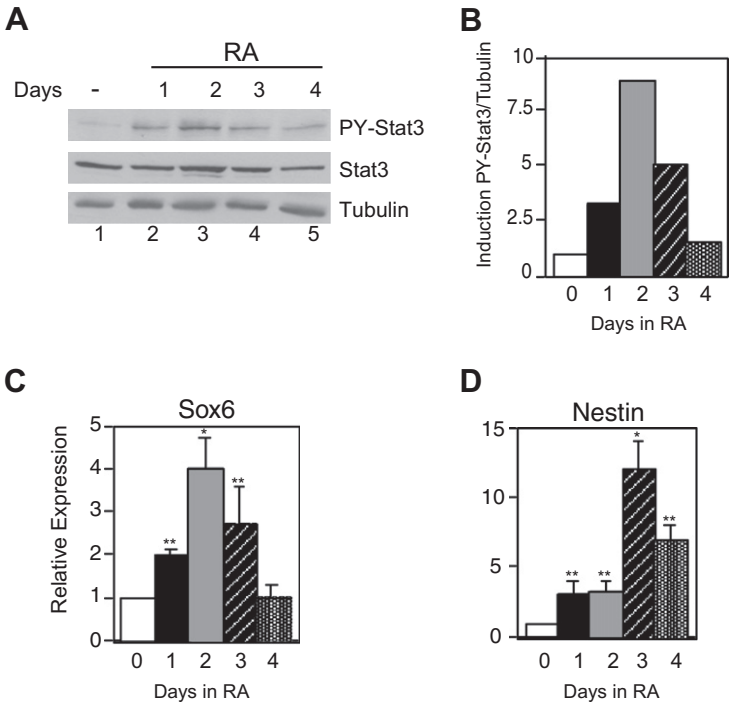

Fig. 3. Stat3 is activated and Sox6 expression is induced during neuronal differentiation of P19 cells. (A) P19 cells were cultured in bacteriological petri dishes either without retinoic acid (RA) or in medium supplemented with 500 nm RA for 1–4 days. Whole cell extracts were analyzed by Western blot using antibodies against Stat3 phosphorylated on tyrosine 705 (PY-Stat3), total Stat3, and tubulin. (B) Results from (A) were quantitated with a Kodak 1D imaging system. Quantitative real-time RT-PCR and statistical analyses were performed for Sox6 (C) or nestin (D) as described in Section 2.

3. Results

3.1. Sox6 is a direct target gene of Stat3

To understand how Stat3 mediates various cellular functions, we performed a genome-wide ChIP screen to search for potential direct Stat3 target genes [8]. One of the genes identified in this screen was Sox6 [8]. To see if the expression of Sox6 is regulated in response to ligands that activate Stat3, NIH3T3 cells were treated with OSM or LIF both of which rapidly induced tyrosine phosphorylation of Stat3 (Fig. 1A and C). Sox6 mRNA expression increased after 2 h of OSM or LIF treatment and was further induced about ninefold by 4 h (Fig. 1B and D).

To demonstrate that Stat3 directly binds to the Sox6 promoter in response to cytokine treatment, gene-specific ChIP analysis was performed with primers flanking a potential Stat3 binding site in the Sox6 promoter. In OSM-treated cells, there was a significant increase in Stat3 binding to the Sox6 promoter compared to untreated cells (Fig. 1E and F).

These results show that in response to ligand stimulation, activated Stat3 binds to the Sox6 promoter and induces transcription of the Sox6 gene.

3.2. LIF increases Sox6 mRNA expression in P19 neuronal cells

It has been shown previously that Sox6 expression is required for neuronal differentiation [9,10]. Furthermore, LIF and Stat3 have been shown to promote neuronal differentiation of mouse ES cells and P19 cells, and inhibition of Stat3 signaling by either JAK2 inhibitors or a dominant negative Stat3 blocks neuronal differentiation [5–7].

To further elucidate the physiological role of Stat3-mediated transcriptional regulation of the Sox6 gene, P19 cells were utilized for further analysis. P19 cells proliferate in growth medium and differentiate into neurons in growth medium supplemented with retinoic acid, thus providing an excellent cellular model system for transcriptional analysis during neuronal differentiation [12]. P19 cells were first cultured in growth conditions and treated with LIF. Stat3 was phosphorylated after 15 min (Fig. 2A) and Sox6 mRNA expression increased by eightfold after 2 h of ligand treatment (Fig. 2B). Because the activation of the JAK-STAT signaling pathway is transient [8,11], the induction of gene expression usually stops after several hours as seen in this case of Sox6 expression in response to LIF at 4 h (Fig. 2B). These results demonstrate that in response to LIF treatment, Stat3 is activated and Sox6 expression is induced in P19 cells. Furthermore, the induction was faster than in NIH3T3 cells.

3.3. Stat3 is phosphorylated during neuronal differentiation of P19 cells and Sox6 expression is increased

It has been shown previously that Sox6 expression increases when P19 cells are cultured in differentiation conditions containing retinoic acid [9]. To determine if Stat3 has a physiological role in Sox6 mRNA expression during neuronal differentiation independent of exogenous ligand stimulation, P19 cells were cultured in the presence of retinoic acid. There was a very low level of phosphorylated Stat3 in cells cultured without retinoic acid (Fig. 3A lane 1 and 3B). Phosphorylated Stat3 levels increased significantly by 2 days in the presence of retinoic acid (Fig. 3A lane 3 and 3B). Similar to the observed pattern of Stat3 phosphorylation, Sox6 mRNA levels increased significantly by 2 days in differentiation conditions (Fig. 3C). As a positive control for efficient neuronal differentiation, nestin mRNA expression was also analyzed because it has been shown previously that nestin levels increase during P19 neuronal differentiation [13]. Nestin levels increased over time in retinoic acid (Fig. 3D). These results suggest that Stat3 is activated during neuronal differentiation of P19 cells independent of exogenous

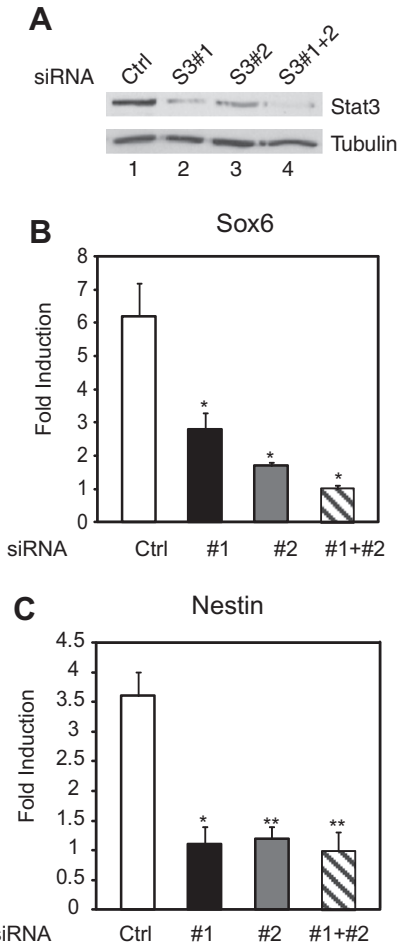

Fig. 4. Stat3 is required for Sox6 expression during neuronal differentiation. P19 cells were transfected with a negative control siRNA, either Stat3 siRNA oligonucleotide (#1 or #2), or both together (#1 + #2) and cultured for 24 h in growth medium. Cells were then transferred to bacteriological petri dishes in the presence or absence of RA for 2 days. Some of the cells cultured in RA were subjected to Western blot analyses and blotted with antibodies against Stat3 or tubulin (A). Quantitative real-time RT-PCR and statistical analyses were performed for Sox6 (B) or Nestin (C) as described in Section 2.

ligand treatment, and Stat3 phosphorylation correlates with an increase in Sox6 expression.

3.4. Stat3 is essential for Sox6 expression during neuronal differentiation

To demonstrate that Stat3 is required for Sox6 expression during neuronal differentiation of P19 cells, we utilized a RNAi technique to knock down Stat3. Two separate siRNAs targeting two different regions of Stat3 [8,11] were used to knock down Stat3 in P19 cells cultured in retinoic acid. To demonstrate that these two siRNAs were specific for Stat3, cells were transfected with a control siRNA or the Stat3 siRNAs separately or combined (#1, #2, or #1 plus #2). Stat3 protein levels were significantly decreased in cells transfected with either Stat3 siRNA compared to cells transfected with a negative control siRNA (Fig. 4A lanes 1, 2, and 3). Stat3 protein levels were further decreased in cells transfected with both Stat3 siRNAs (#1 plus #2) (Fig. 4A lane 4). These results demonstrate that the Stat3 siRNAs specifically and efficiently knocked down Stat3 protein levels in P19 cells.

Sox6 mRNA expression was analyzed in the Stat3 knockdown cells. Cells transfected with a negative control siRNA showed a six-fold increase in Sox6 expression when cultured in retinoic acid (Fig. 4B). In cells transfected with either Stat3 siRNA (#1 or #2) and cultured in retinoic acid, induction of Sox6 was about half compared to control cells (Fig. 4B). Sox6 expression was not significantly induced in cells transfected with both Stat3 siRNAs (#1 plus #2) at the same time (Fig. 4B). As a positive control for neuronal differentiation, nestin expression was also monitored (Fig. 4C). Nestin levels were not significantly induced in cells transfected with either Stat3 siRNA (#1 or #2) or both (#1 plus #2) compared to cells transfected with a control siRNA. These results demonstrate that Stat3 is required for Sox6 mRNA expression during neuronal differentiation of P19 cells.

3.5. Stat3 is essential for neuronal differentiation in P19 cells

To demonstrate that Stat3 is essential for neuronal differentiation, Stat3 protein levels were knocked down during retinoic acid-induced differentiation (Fig. 5A) and cell morphology was analyzed 7 days post-differentiation. Cells transfected with a control siRNA and cultured in retinoic acid showed efficient neurite outgrowth while Stat3 knockdown cells showed no significant neuronal differentiation (Fig. 5B). Two neuronal differentiation markers, N-cadherin and NCAM, were significantly induced over time in control cells but not induced in the Stat3 knockdown cells (Fig. 5C and D). These results demonstrate that Stat3 is required for efficient neuronal differentiation of P19 cells.

4. Discussion

This work demonstrates that Stat3 binds to the promoter and activates transcription of the Sox6 gene in response to ligand treatment. Furthermore, Stat3 increases Sox6 expression in P19 cells in response to cytokine treatment and during neuronal differentiation independent of exogenous ligand. Finally, Sox6 expression is dependent on Stat3 during neuronal differentiation of P19 cells. These results identified a molecular mechanism by which Stat3 promotes neuronal differentiation through transcriptional regulation of Sox6.

Sox6 is not the only gene regulated by Stat3 during neuronal differentiation. It has been shown previously that Stat3 regulates expression of another member of the SOX family during neuronal differentiation, Sox2 [5]. Stat3 could regulate transcription of other members of the SOX gene family or other genes to promote neuronal differentiation. Because of the multi-step nature of neuronal differentiation, we cannot determine exactly at which step Stat3 functions. It remains to be studied whether Stat3, usually activated in immediate early responses, plays an important physiological role specifically in the early stages of neuronal differentiation.

It is interesting to note that Stat3 regulates transcription during other cellular differentiation processes. In addition to promoting neuronal differentiation, Stat3 also promotes cardiac muscle cell differentiation [11,14,15]. In contrast, Stat3 inhibits skeletal muscle cell differentiation [8,16]. These observations suggest that Stat3 could be an important regulator of cellular differentiation and development. Further studies of Stat3 and its target genes will provide greater insights into the mechanisms by which Stat3 controls cellular differentiation.

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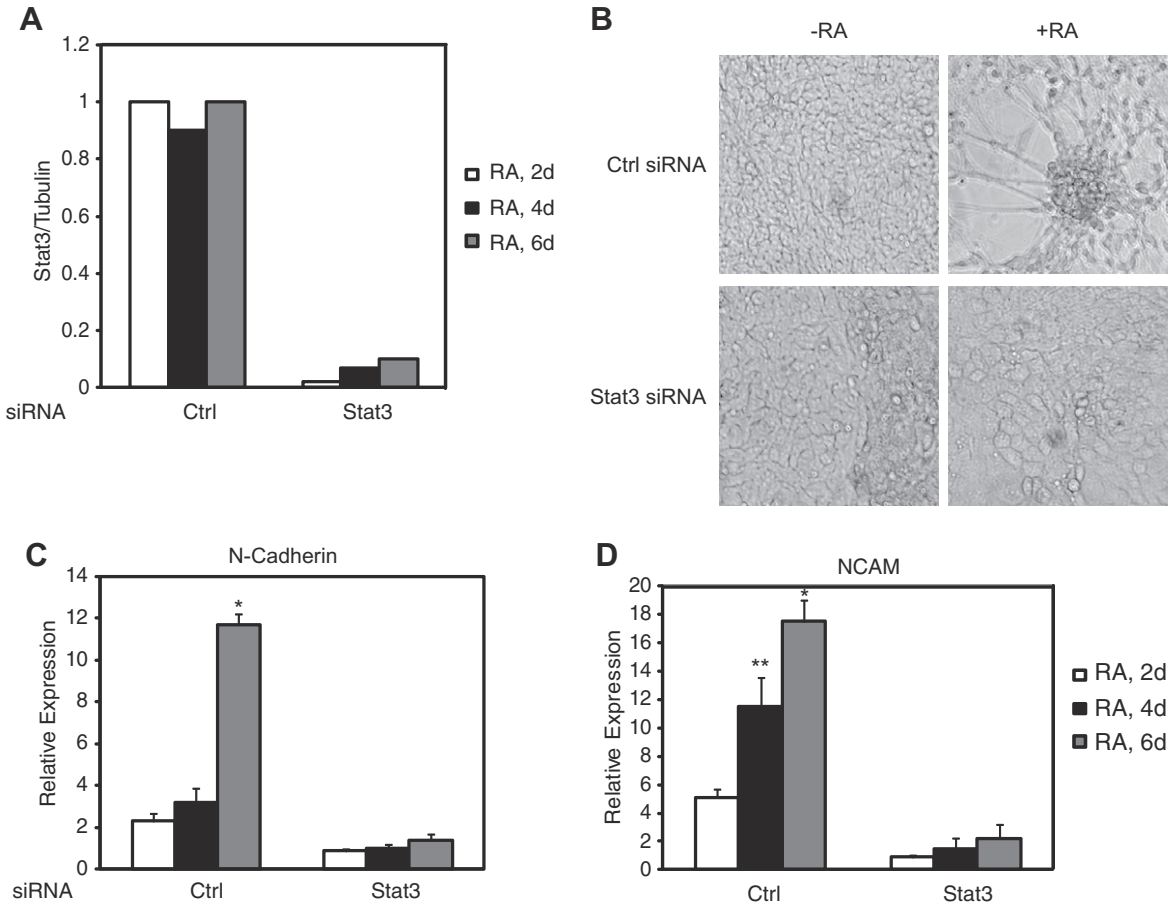

Fig. 5. Stat3 is required for efficient neuronal differentiation. P19 cells were transfected with a negative control siRNA or both Stat3 siRNAs and cultured for 24 h in growth medium. Cells were then transferred to bacteriological petri dishes in the presence or absence of RA for 4 days and transfected a second time with indicated siRNAs. Cells were transferred to tissue culture dishes and cultured for a total of 7 days. (A) Western blot analyses were performed at 2, 4 and 6 days, blotted with Stat3 and tubulin antibodies, quantitated and expressed as Stat3/tubulin. (B) Cells were examined microscopically at 7 days for neuronal differentiation. (C and D) Quantitative real-time RT-PCR and statistical analyses were performed as described in Section 2 for N-cadherin and NCAM at indicated time points.

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